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(57) Abstract

Described herein are two novel members of the Sterile 20 family of serine/threonine protein kinases.

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# TWO STERILE-20 KINASE-LIKE PROTEINS AND METHODS OF USE THEREOF

## RELATED APPLICATION

This application claims benefit of U.S. Provisional application 60/069,078, filed December 9, 1997 the entire teachings of which are incorporated herein by reference.

## BACKGROUND

Protein kinases play a key role in cell growth and differentiation. The p21-activated proteins kinases (PAKs) are related to a yeast serine/threonine protein kinase, Ste20. Ste20 is a member of a growing family of regulatory enzymes that may play roles in diverse phenomena such as cellular morphogenesis, the stress response and the pathogenesis of AIDS.

The growth, differentiation, maintenance and senescence of cells requires the transmission of signals. These signals can be derived from extracellular stimuli, such as hormone interaction with its receptor or physiological stress, or may be derived intracellularly from developmental programs.

The signals are transmitted via a signal cascade wherein proteins are phosphorylated or dephosphorylated in sequence. Protein kinases, the enzymes that phosphorylate, play a key role in many signaling pathways and therefore protein kinases play a key role in cell growth and differentiation. Protein kinases fall into two broad categories, tyrosine kinases (those that add phosphate to tyrosine residues) and serine/threonine kinases (those that add phosphate to either serine or threonine residues).

One group of signaling pathways involving protein kinases are the mitogen-activated protein (MAP) kinase cascades. The MAP kinase cascades are now recognized to participate in diverse signal transduction pathways rather than only

Genetic analyses in yeast have made it possible to identify some of the components of MAPK pathways, and to clarify the diversity of their functions and regulation (Herskowitz, I., 1995, *Cell* 80:187-197). Mammalian cells have at least three MAPK pathway subtypes, the ERK (extracellular signal-regulated protein kinase) pathway, JNK (c-Jun $NH_2$-terminal kinase)/SAPK (stress-activated protein kinase) pathway, and P38/Mpk2 pathway; together, these pathways mediate a wide variety of physiological responses (Davis, R.J., 1994, *Trends in Biochem. Science* 19:470-473; Derijard, B. *et al.*, 1994 *Cell* 76:1025-1037; Kyriakis, J.M. *et al.*, 1994 *Nature* 369:156-160; Han, J. *et al.*, 1994 *Genes Dev.* 3:1336-1348; Lee, J.C. *et al.*, 1994 *Nature* 372:739-746; Rouse, J. *et al.*, 1994 *Cell* 78:1027-1037). MAPKs are activated by sequential protein phosphorylation reactions. The basic framework of the MAP kinase pathway, where MAPK is phosphorylated on Thr and Tyr residues and activated by MAPK kinase (MAPKK), after which MAPKK is itself phosphorylated and activated by MAPKK kinase (MAPKKK), is common from yeast to mammals (Nishida, E. and Gotoh, Y., 1993 *Trends in Biochem. Science* 18:128-131; Davis, 1994; Herskowitz, 1995; Marshall, C.J., 1994 *Curr. Opin. Genet. Dev.* 4:82-89).

A new kinase group activated by G-protein and thought to act as MAPKKK kinase (MAPKKKK) has been identified in both yeast and mammals. In budding yeast, this new kinase group is known by its prototype member Sterile 20 (Ste20). Ste20 is activated by the βγ complex released from the heterotrimeric G protein complex upon pheromone receptor stimulation, and in turn activates Ste11 (a MAPKKK) (Leberer, E. *et al.*, 1992 *EMBO J.* 11:4815-4824; Ramer, S.W. and Davis, R.W., 1993 *Proc. Natl. Acad. Sci. U.S.A.* 90:452-456). In mammals, this new kinase group is known by its prototype member p21-activated protein kinase (PAK). PAK (now called α-PAK) has been identified as a protein kinase activated by the Rho family of small G-proteins, Rac1 and Cdc42 (Manser, E. *et al.*, 1994 *Nature* 367:40-46); PAK also shows sequence similarity to yeast Ste20. Recently it has been clarified that PAK comprises a protein kinase family composed of several PAK

isoforms, hPAK65 (Martin, G.A. *et al.*, 1995 *EMBO J.* 14:1970-1978), MPAK-3 (Bagrodia, S. *et al.*, 1995 *J. Biol. Chem.* 270:22731-22737 and δ-PAK (Manser, E. *et al.*, 1995 *J. Biol. Chem.* 270:25070-25078), all of which are able to interact with Cdc42 and Rac1. Rac1 and Cdc42 have been implicated not only in cell motility (Ridley, A.J. *et al.*, 1992 *Cell* 70:401-410; Kozma, R. *et al.*, 1995 *Mol. Cell. Biol.* 15:1842-1952; Nobes, C.D. and Hall, A., 1995 *Cell* 81:53-62), but also in the preferential activation of the JNK/SAPK and p38/Mpk2 pathways rather than the ERK pathway (Coso, O.A. *et al.*, 1995 *Cell* 81:1137-1146; Minden, A. *et al.*, 1995 *Cell* 81:1147-1157; Olson, M.F. *et al.*, 1995 *Science* 269:1270-1272). This is in contrast to another small G-protein, Ras, which predominantly activates the ERK pathway through Raf activation (Minden, A. *et al.*, 1994 *Science* 266:1719-1723). Although a direct interaction between PAKs and components of the JNK/SAPK and p38/Mpk2 pathways has not yet been demonstrated, these observations raise the intriguing possibility that PAK or PAK-related proteins mediate the signals from Rac1 and Cdc42 to the JNK/SAPK and p38/Mpk2 pathways, and, furthermore, that G-proteins differentially regulate MAPK pathways to achieve various physiological responses.

In contrast to the above-mentioned kinases, a group of Ste20-related kinases that lack the putative Cdc42/Rac1-binding domain has been identified in both yeast and mammals: Sps1, an upstream regulator of the MAPK pathways (Creasy, C.L. and Chernoff, J., 1995 *J. Biol. Chem.* 270:21695-21700). Although the specific activation of the SAPK pathway by BCK has recently been reported (Pombo, C.M. *et al.*, 1995 *Nature* 377:750-754), the upstream and downstream signaling pathways of this group of kinases remain to be clarified. Sterile 20-related kinases are regulatory molecules involved in mitogenic signaling as well as other cellular phenomena such as morphology and motility. These phenomena are important factors in development, cell differentiation, cancer and metastases. Therefore, the polynucleotides and polypeptides of the present invention allow manipulation of the signaling pathways involved and will allow the development of reagents to modulate the signaling pathways involved in these important cellular phenomena.

## SUMMARY OF THE INVENTION

The present invention relates to isolated polynucleotides encoding novel members of the Ste20 family of serine/threonine protein kinases, and the recombinantly produced polypeptides encoded by said polynucleotides.

The present invention is drawn to a purified nucleic acid (also referred to herein as a polynucleotide) comprising at least 10 continuous nucleotides of a nucleic acid sequence provided in SEQ ID NO.: 1. The present invention is drawn to a purified nucleic acid comprising at least 10 continuous nucleotides of a nucleic acid sequence provided in SEQ ID NO.: 3. The present invention is also drawn to a purified nucleic acid sequence comprising a nucleic acid sequence encoding at least 8 continuous amino acids of a amino acid sequence provided in SEQ ID NO.: 2.

Also encompassed by the present invention are the following: a purified nucleic acid sequence comprising a nucleic acid sequence encoding at least 8 continuous amino acids of a amino acid sequence provided in SEQ ID NO.: 4; a purified nucleic acid comprising a nucleotide sequence which comprises at least 16, 18, 19 or 20 nucleotides, hybridizing under stringent conditions, to at least 20 continuous nucleotides provided in SEQ ID NO. 1; a purified nucleic acid comprising a nucleotide sequence which comprises at least 16, 18, 19 or 20 nucleotides, hybridizing under stringent conditions, to at least 20 continuous nucleotides provided in SEQ ID NO. 3; a purified nucleic acid comprising SEQ ID NO. 1 or SEQ ID NO. 3.

The present invention also encompasses expression vectors comprising the polynucleotides of the present invention and host cells harboring said vectors. The present invention is also drawn to a purified nucleic acid comprising a nucleic acid sequence encoding an amino acid sequence comprising SEQ ID NO. 2 or a nucleic acid sequence encoding an amino acid sequence comprising SEQ ID NO. 4, and to a method of making polypeptides encoded by SEQ ID NO.1 or SEQ ID NO. 3 comprising, transfecting a host cell with an expression vector comprising SEQ ID NO. 1 or SEQ ID NO. 3 and isolating the expressed protein.

The present invention is further drawn to polypeptides comprising SEQ ID NO. 2 or SEQ ID NO. 4 and biologically active fragments thereof; polypeptides functionally equivalent to polypeptides comprising SEQ ID NO. 2 or SEQ ID NO. 4 or fragments thereof; antibodies that bind to polypeptides encoded by SEQ ID NO.1 and antibodies that bind to polypeptides encoded by SEQ ID NO. 3.

The present invention is further drawn to a serine/threonine kinase comprising the amino acid sequence of SEQ ID NO. 2, and to a serine/threonine kinase comprising the amino acid sequence of SEQ ID NO. 4.

The protein kinase described herein has homology with a family of proteins that appear to function in the mitogen-activated protein (MAP) kinase cascade. The present invention also relates to methods of using the polynucleotides and polypeptides described herein to detect, isolate and characterize elements upstream and downstream of the novel kinase in the signal transduction pathway using assays well known in the art such as kinase assays or co-immunoprecipitation assays, or combinations thereof. Furthermore, polypeptides of the present invention include biologically active fragments of the proteins described herein. Such proteins and biologically active fragments are useful to generate antibodies that specifically bind the proteins of the present invention. The biologically active fragments are also useful as tools to study the activity of the protein. Altered forms of the polypeptides are within the scope of the present invention and can be used to study the activity of downstream elements in the signaling pathway or to generate specific antibodies.

The isolated polynucleotides and polypeptides of the present invention provide the advantage of being able to conveniently manipulate the genes, gene products and expression level of the gene product, to facilitate understanding of how Ste20 and Ste20 family members regulate signal transduction in the cell.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the cDNA sequence encoding C12.2bs (SEQ ID NO. 1).

Figures 2A and 2B depicts the cDNA sequence encoding 5e.new (SEQ ID NO. 3).

Figure 3 depicts the PCR primers (SEQ ID NOs. 5-7) used to clone the novel genes of the present invention.

Figure 4 depicts the amino acid sequence of C12-2bs (SEQ ID NO. 2), 5e. new (SEQ ID NO. 4) and S201 (SEQ ID NO. 8), a human Ste20 homologue.

Figure 5 depicts an amino acid sequence alignment between C12.2bs (SEQ ID NO. 2) and 5e.new (SEQ ID NO. 4).

## DETAILED DESCRIPTION OF THE INVENTION

Ste20/PAK serine/threonine protein kinases (referred to herein as Sterile 20 protein kinases or Ste20) have been suggested as playing essential roles in diverse phenomena such as cellular morphogenesis, the stress response and the pathogenesis of AIDS. Recently, mammalian Ste20 family members have been discovered that do not appear to participate in the three known MAP kinase cascades. While much is known about the Ste20 family of protein kinases and their interaction with the MAP kinase cascade, it is clear that the full extent of the Ste20 family as well as the upstream and downstream regulatory components are poorly understood.

Described herein are new members of the Ste20 family that were cloned from a murine cDNA library using polymerase chain reaction (PCR). Degenerate primers SEQ ID NO. 5, SEQ ID NO. 6 and SEQ ID NO. 7 (Figure 3) were used in the following combinations: SEQ ID NO. 5 with SEQ ID NO. 6, and SEQ NO. 5 with SEQ ID NO. 7, in a standard PCR reaction as described in the Exemplification. Novel members of the Ste20 family were isolated and are shown in Figures 1 and 2 (SEQ ID NO. 1 and SEQ ID NO. 3).

The present invention encompasses the isolated and/or recombinant nucleic acid sequences encoding novel members of the Sterile 20 family of serine/threonine protein kinases, functional equivalents thereof or biologically active fragments

thereof, as described herein. The present invention further encompasses sequences complementary to, or homologous with SEQ ID NO. 1 and SEQ ID NO. 3.

The polynucleotides of the present invention, or portions thereof, can be used as probes to isolate and/or clone substantially similar or functionally equivalent homologues of the Ste20 family of proteins. The polynucleotides of the present invention can also be used as probes to detect and or measure expression of the genes encoded by the present invention. Expression assays, such as Southern blot anaysis and whole mount *in situ* hybridization are well known in the art. The polynucleotides of the present invention, or portions thereof, can be used as primers to clone homologues or family members by PCR using techniques well known in the art.

As used herein, nucleic acids are also referred to as DNA and RNA, or DNA sequences and RNA sequences, or DNA molecules or RNA molecules. Nucleic acids referred to herein as "isolated" are nucleic acids separated away from the nucleic acids of the genomic DNA or cellular RNA of their source of origin (e.g., as it exists in cells or in a mixture of nucleic acids such as a library), and may have undergone further processing. "Isolated" nucleic acids include nucleic acids obtained by methods known to those of skill in the art to obtain isolated nucleic acids and methods described herein. These isolated nucleic acids include essentially pure nucleic acids, nucleic acids produced by chemical synthesis, by combinations of biological and chemical methods, and recombinant nucleic acids produced by recombinant methods, which are well known in the art.

Nucleic acids referred to herein as "recombinant" are nucleic acids which have been produced by recombinant DNA methodology, including those nucleic acids that are generated by procedures which rely upon a method of artificial recombination, such as the polymerase chain reaction (PCR) and/or cloning into a vector using restriction enzymes. "Recombinant" nucleic acids are also those that result from recombination events that occur through the natural mechanisms of cells, but are selected for after the introduction to the cells of nucleic acids designed to allow and make probable a desired recombination event.

Also encompassed by the present invention are nucleic acid sequences (DNA or RNA sequences) which are complementary, substantially homologous to, or functionally equivalent to the Sterile 20 protein kinase DNA sequences described herein. Fragments of the sequences, complementary sequences, substantially homologous sequences and functionally equivalent sequences are also encompassed by the present invention. Nucleic acid sequences hybridizing with sequences comprising SEQ ID Nos 1 and 3 and portions thereof under conditions of stringency known to those of skill in the art to be sufficient to identify DNA sequences with substantial nucleic acid sequence identity are also encompassed by the present invention. Due to the degeneracy of the genetic code, different combinations of nucleotides can encode for the same polypeptide or the homologous polypeptide in a different organism. Thus, different nucleic acids can encode the same Sterile 20 protein or Sterile 20 homologue. These polynucleotides are referred to herein as functionally equivalent polynucleotides. In preferred embodiments, substantially homologous polynucleotides of the present invention comprise at least 10, at least 27, at least 45 or at least 102 continuous nucleotides of SEQ ID NO.: 1 or SEQ ID NO.: 3. It is reasonable to predict that DNA sequences identified under such stringent conditions will likely encode a protein (also referenced to herein as a polypeptide, or peptide fragment) with the biological activity or physical characteristics of Sterile 20 protein kinases comprising SEQ ID NOs 2 or 4. The nucleic acid of the present invention preferably encodes 8, 18, 34 or 54 continuous amino acids of the amino acid sequence provided in SEQ ID NO. 2 or the amino acid sequence provided in SEQ ID NO. 4.

Polypeptides with the biological activity or physical characteristics of Sterile 20 protein kinases comprising SEQ ID NOs 2 or 4 are referred to herein as functionally equivalent polypeptides. "Functional or biologically active protein" is defined herein as a protein which shares significant identity (e.g., at least about 65%, preferably at least about 80% and most preferably at least about 95%) with the corresponding sequences of the endogenous protein and possesses one or more of the functions thereof.

"Polypeptide" as used herein indicates a molecular chain of amino acids and does not refer to a specific length of the product. Thus, peptides, polypeptides and proteins are included within the definition of polypeptide. This term is also intended to refer to post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like.

A general description of stringent hybridization conditions are discussed in Ausubel, F.M., *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience 1989, the teachings of which are incorporated herein by reference. Factors such as probe length, base composition, percent mismatch between the hybridizing sequences, temperature and ionic strength influence the stability of nucleic acid hybrids. Thus, stringency conditions sufficient to identify the polynucleotides of the present invention, (e.g., high or moderate stringency conditions) can be determined empirically, depending in part upon the characteristics of the known DNA to which other unknown nucleic acids are being compared for sequence similarity.

Alternatively, conditions for stringency are as described in WO 98/40404, the teachings of which are incorporated herein by reference. In particular, examples of highly stringent, stringent, reduced and least stringent conditions are provided in WO 98/40404 in the Table on page 36. In one embodiment, highly stringent conditions are those that are at least as stringent as, for example, 1x SSC at 65°C, or 1x SSC and 50% formamide at 42°C. Moderate stringency conditions are those that are at least as stringent as 4x SSC at 65°C, or 4x SSC and 50% formamide at 42°C. Reduced stringency conditions are those that are at least as stringent as 4x SSC at 50°C, or 6x SSC and 50% formamide at 40°C.

As defined herein, substantially complementary means that the sequence need not reflect the exact sequence of e.g., SEQ ID NOs. 1 or 3, but must be sufficiently similar in identity of sequence to hybridize with SEQ ID NOs. 1 or 3 under stringent conditions. For example, non-complementary bases, or longer or shorter sequences can be interspersed in sequences, provided the sequence has sufficient complementary bases with, e.g., SEQ ID NOs. 1 or 3 to hybridize therewith. Under

stringent hybridization conditions, only highly complementary nucleic acid sequences hybridize. Preferably, such conditions prevent hybridization of nucleic acids having four mismatches out of 20 continuous nucleotides, more preferably two mismatches out of 20 continuous nucleotides, most preferably one mismatch out of 20 continuous nucleotides.

Biological functions of the Sterile 20 kinase proteins include phosphorylation of serine and threonine residues in response to extracelluarly derived or intracellularly derived stimuli. Phosphorylation activity can be measured using methods well know in the art. For example, the kinase of the present invention can be epitope-tagged using methods well known in the art. The tagged protein are then expressed in suitable cells, such as COS7 cells, using methods well known in the art. The cells are then stimulated or not, to activated the Sterile 20 signal transduction pathway and lysed under appropriate conditions. The kinase is isolated by immunoprecipitation. The kinase activity is measured in a standard kinase assay with a suitable substrate, such as myelin basic protein (see, for example, Creasy and Chernoff, *J. Biol. Chem. 270*:21695-21700 (1995)). The present invention also pertains to an isolated nucleic acid sequence encoding an altered kinase protein, wherein the kinase has altered activity. The activity can be enhanced or reduced or abolished. The alteration can result in a constitutively active kinase. In one embodiment, the resulting alteration in amino acid sequence is in the catalytic domain of the kinase.

Biological activity of the present invention further includes the ability to bind the normal upstream (for example an activator) or downstream (for example the target to be phosphorylated) element in the signaling pathway. Also described herein, biological activity can include the antigenicity of the protein, or peptide, resulting in the production of antibodies which bind to the polypeptides, Sterile 20 kinases and serine/threonine kinases of the present invention.

The present invention also relates to methods of altering the biologial activity of the polypeptides of the present invention. Alteration can be, for example, an increase or decrease in serine/threonine kinase activity, alteration in cellular

localization of the protein or alteration in interaction of the polypeptides of the present invention with upstream or downstream elements of the signaling pathway.

The present invention is understood to include the polypeptides having amino acid sequences comprising SEQ ID NO. 2 or SEQ ID NO. 4. The present invention includes polypeptides comprising amino acid sequences analogous to SEQ ID NO. 2 or SEQ ID NO. 4. Such proteins are defined herein as C12-2bs or 5e.new analogs, or C12-2bs or 5e.new variants. Analogous amino acid sequences are defined herein to mean amino acid sequences with sufficient identity of amino acid sequence with, e.g., C12-2bs or 5e.new proteins, to possess the biological activity of Sterile 20 protein kinase. The biological activity of Sterile 20 protein kinase can include, for example, the capability to phosphorylate the same target as that phosphorylated by 5e.new or C12-2bs such as serine and threonine residues of target proteins in human and animal cells in response to extracelluarly derived or intracellularly derived stimuli. For example, an analog polypeptide can be produced with "silent" changes in the amino acid sequence wherein one, or more amino acid residue differs from the amino acid residues of C12-2bs or 5e.new, yet still possess the biological activity of 5e.new or C12-2bs. Examples of such differences include additions, deletions or substitutions of residues to e.g., SEQ ID NOs. 2 or 4. Also encompassed by the present invention are variant proteins that exhibit lesser or greater biological activity of the Sterile 20 protein kinases of the present invention.

Variant proteins of the present invention can be produced using *in vitro and in vivo* techniques well-known to those of skill in the art, for example, site-specific mutagenesis and oligonucleotide mutagenesis. Manipulations of the protein sequences can be made a the protein level as well. Any numerous chemical modifications can be carried out by known techniques including, but not limited to, specific chemical cleavage by cyanogen bromide, trypsin and papain. The proteins of the present invention can also be structurally modified or denatured, for example, by heat. In general, mutations can be conservative or non-conservative amino acid substitutions, amino acid insertions or amino acid deletions.

For example, DNA encoding a variant protein of the present invention is prepared by site-directed mutagenesis of the polynucleotides comprising SEQ ID NO. 1 or SEQ ID NO. 3. Site-directed (site-specific) mutagenesis allows the production of protein variants through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. The term "primer" denotes a specific oligonucleotide sequence complementary to a target nucleotide sequence and used to hybridize to the target nucleotide sequence and serve as an initiation point for nucleotide polymerization catalyzed by either DNA polymerase, RNA polymerase or reverse transcriptase. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the techniques of site-specific mutagenesis are well known in the art, as exemplified by publications such as Edelman *et al.*, *DNA 2:*183, 1983. The site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing *et al.*, *Third Cleveland Symposium on Macromolecules and Recombinant DNA*. A. Walton, ed., Elsevier, Amsterdam, 1981. This and other phage vectors are commercially available and their use is well-known to those skilled in the art. A versatile and efficient procedure for the construction of oligonucleotide directed site-specific mutations in DNA fragments using M13-derived vectors was published by Zoller, M.J. and Smith, M., *Nucleic Acids Res. 10*:6487-6500, 1982. Also, plasmid vectors that contain a single-stranded phage origin of replication can be employed to obtain single-stranded DNA. Veira *et al.*, *Meth Enzymol. 153*:3 1987. Alternatively, nucleotide substitutions can be introduced by synthesizing the appropriate DNA fragment *in vitro*, and amplifying it by PCR procedures known in the art.

In general, site-specific mutagenesis herewith can be performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence

that encodes the relevant protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea *et al.*, *Proc Natl Acad Sci USA. 75:*5765, 1978. This primer can then be annealed with the single-stranded protein sequence-containing vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector can then be used to transform appropriate host cells such as JM 101 cells, and clones can be selected that include recombinant vectors bearing the mutated sequence arrangement. Thereafter, the mutated region can be removed and placed in an appropriate expression vector for protein production.

The PCR technique can also be used in creating amino acid sequence variants of the proteins of the present invention. When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers can be designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer is preferably identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 500 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the end position of the mutation specified by the primer.

The DNA fragments produced bearing the desired mutation can be used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced

simultaneously by either using a mutant second primer or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more) part ligation.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al. Gene* 34, 315, 1985. The starting material can be the plasmid (or vector) comprising SEQ ID NO. 1 or SEQ ID NO 3, or a portion thereof to be mutated. The codon(s) within the polynucleotide to be mutated are identified. There must be unique restriction endonuclease sites on each side of the identified mutation site(s). If such restriction sites do not exist, they can be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the polynucleotide. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. The plasmid now contains the mutated DNA sequence, that can be expressed to produce altered proteins with altered kinase, binding or antigenic activity.

The present invention also encompasses biologically active protein, or biologically active fragments thereof as described herein. Such fragments can include part of the full-length amino acid sequence of a Sterile 20 protein kinase yet possess biological activity. Such fragments can be produced by amino- and carboxyl-terminal deletions, as well as internal deletions. Such peptide fragments can be tested for biological activity as described herein. Thus, a functional or biologically active protein includes mutants or variants of the endogenous protein wherein one or more amino acids have been substituted, deleted or added. The biologically active fragments can be altered to have enhanced or reduced biological activity.

The biologically active fragments of the protein and polypeptides of the present invention comprise at least six continuous amino acids provided in SEQ ID. NOs.: 2 and 4. The proteins and polypeptide fragments can be used, for example, for structure determination, to assay other molecules' effects on the activity of the polypeptides of the present invention. These effects can be to inhibit or enhance the biological activity or to alter the subcellular location of the protein. The proteins and polypeptide fragments of the present invention can further be used to obtain antibodies that specifically bind to the polypeptide of the present invention. A useful fragment of the polypeptide of interest comprises about 5 to about 35 amino acids of the polypeptides of the present invention.

The DNA sequences of the present invention can also be used in a recombinant construct for the infection, transfection or transformation of a cell *in vitro* or *in vivo* under control of an appropriate promoter for the expression of functional Sterile 20 protein kinases, as defined herein, in an appropriate host cell. Such recombinant constructs are also referred to herein as expression vectors. For example, a DNA sequence can be functionally ligated to a suitable promoter (e.g., a constitutive or inducible promoter or the endogenous promoter) introduced into a suitable expression vector which is then introduced into a suitable host cell. Examples of useful promoter sequences include, for example, the early and late promoters of SV40 or adenovirus, the T3 and T7 promoters, the major operator and promoter regions of phage lambda, and the like. Constitutive and inducible promoter sequences known to control the expression of genes in prokaryotic or eukaryotic cells their viruses or various combinations thereof are useful in the expression of the DNA sequences of the present invention. Suitable host cells for use in expressing the DNA sequences of the present invention include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, insect cells such as *Spodoptera frugiperda* (SF9), animal cells such as CHO and mouse cells, African green monkey cells such as COS1 and COS7 and human cells as well as plant cells in tissue culture. One of skill in the art may make a selection among the vectors expression control sequences and host cells without undue experimentation

and without departing from the scope of this invention. The construct can also include DNA encoding one or more selectable markers (such as neo, gdhfr and hyg) or DNA encoding one or more different antigens or therapeutic proteins.

The invention also provides vectors containing the serine/threonine kinases of the present invention and altered forms thereof. Suitable vectors for use in eukaryotic and prokaryotic cells are well known in the art and are, generally commercially available, or readily prepared by the skilled artisan. For example, suitable plasmids for use include pGEX 2T/3X/4T or pET series vectors. Additional vectors can also be found in, for example, Ausubel *et al.*, "*Current Protocols in Molecular Biology*", John Wiley & Sons, (1998) and Sambrook *et al.*, "*Molecular Cloning: A Laboratory Manual*", 2nd Ed. (1989), the teachings of which are incorporated herein by reference.

The construct can be introduced by any suitable means, as set forth above, such as by calcium phosphate precipitation, microinjection, electroporation or infection (such as with an infectious retroviral, herpes vaccinia or adenovirus vector). The host cell can be a eucaryotic or procaryotic cell. Suitable cells include bacterial (e.g. *E. coli*) or mammalian cells. Mammalian cells include primary somatic cells, such as, epithelial cells, fibroblasts, keratinocytes, macrophages or T cells, or immortalized cell lines, such as HeLa or HT1080. The recombinant host cell can then be cultured and, optionally, selected, *in vitro* under appropriate conditions resulting in the expression of the protein. Alternatively, the cell can be transplanted or injected into an animal, such as a human, for *in vivo* expression.

The polypeptides and biologically active fragments thereof of the present invention may be isolated or purified from recombinant cells or tissue expressing said polypeptides and purified using any of a variety of conventional methods. These methods may include, for example, precipitation, such as ammonium sulfate, ethanol, acetone or immuno-precipitation, gel electrophoresis, chromatographic techniques, such as normal or reversed phase liquid chromatography, HPLC, FPLC, affinity chromatography and size exclusion chromatography or a combination thereof. "Isolated or purified polypeptide" means a polypeptide of interest or fragment thereof

which is essentially free, that is, contains less than about 50%, preferably less than about 70%, and more preferably, less than about 90% of cellular components with which the polypeptide of interest is naturally associated. Methods for purifying are known in the art. One of skill in the art may select the most appropriate isolation and purification technique without departing from the scope of this invention.

The present invention further relates to fusion proteins comprising the Sterile 20 protein kinases described herein (referred to herein as a first moiety) linked to a second moiety not occurring in the Sterile 20 protein kinase as found in nature. Thus, the second moiety can be a single amino acid, peptide or polypeptide. The first moiety can be in a biologically active fragment of the polypeptide of the present invention linked at an N-terminal location, a C-terminal location or to the second moiety. The biologically active fragment of the polypeptide of the present invention can be fused at both termini to a second moiety. In one embodiment, the fusion protein comprises a Sterile 20 protein kinase protein and either a maltose binding protein (MBP) or glutathione-S-transferase (GST). In another embodiment, the second moiety is an epitope tag, such as a myc tag or an HA tag. Such fusion proteins or epitope-tagged proteins can be isolated using methods well known in the art and are useful to produce specific antibodies or can be used *in vitro* kinase assays.

Specific antibodies can be used to detect the presence of the polypeptides of the present invention, fragments thereof or altered forms thereof using standard enzyme-linked immunosorbant assay, radioimmunoassay and immunoblot analysis. Specific antibodies of the present invention can also be used for immuno-cytochemistry on cells or tissues. For example, polyclonal and monoclonal antibodies, including non-human and human antibodies, humanized antibodies, chimeric antibodies and antigen-binding fragments thereof (*Current Protocols in Immunology*, John Wiley & Sons, N.Y. (1994); EP Application 173,494 (Morrison); International Patent Application WO86/01533 (Neuberger); and U.S. Patent No. 5,225,539 (Winters)), which bind to the described polypeptide or altered polypeptides or fragments thereof, are within the scope of the invention.

Antibodies of the present invention can be generated, for example, by immunizing a mammal, such as a mouse, rat, hamster or rabbit, with an immunogenic form of the polypeptides of the present invention or altered form thereof that are capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. The protein or polypeptide can be administered in the presence of an adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibody. Following immunization, antisera can be obtained, and if desired, polyclonal antibodies can be isolated from the serum. Monoclonal antibodies can also be produced by standard techniques, which are well known in the art (Kohler and Milstein, *Nature 256*:495-497 (1975); Kozbar et al., *Immunology Today 4*:72 (1983); and Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp. 77-96 (1985)). The term "antibody" as used herein is intended to include fragments thereof, such as Fab and $F(ab)_2$.

Antibodies described herein can be used to inhibit the activity of the phosphatase described herein, particularly *in vitro* and in cell extracts, using methods known in the art. Additionally, antibodies can be detectably labeled, such as with a radioactive label, and used to assay for the presence of the expressed protein in a cell, such as yeast or tissue culture or a tissue sample, and can be used in an immunoabsorption process, such as an ELISA, to isolate the polypeptides or fragments thereof of the present invention or altered forms thereof. Tissue samples which can be assayed include mammalian tissues, e.g., differentiated and non-differentiated cells. Examples include bone marrow, thymus, kidney, liver, brain, pancreas, fibroblasts, epithelium, and muscle.

The novel family members of Sterile 20, C12-2bs (SEQ ID NO. 1) and 5e.new (SEQ ID NO. 3) described herein are useful to study upstream and downstream elements in the signal transduction cascades involving these molecules, homologues of these molecules or family members. Further, SEQ ID NO. 1 and

SEQ ID NO. 3 or portions thereof can be used to isolate and clone homologues of these genes from other organisms such as humans, or to clone closely related family members in mouse or in other organisms such as humans. SEQ ID NO.: 1 and 3, or portions thereof can be used as probes or PCR primers. The proteins encoded by SEQ ID NO. 2 and SEQ ID NO. 4 can be used in standard phosphorylation assays to discover and characterize effectors and substrates of these proteins.

These signaling molecules participate in a diverse set of events in the cell in response to many different stimuli. For example, for this family of kinases, oxidative stress can be a regulatory element. These kinases are important for ischemic, stroke, heart disease, inflammation, and cancer and are targets for drug therapeutics, both for blocking agents as well as agents that enhance kinase activity.

The present invention will now be illustrated by the following example, which are not intended to be limiting in any way.

## EXEMPLIFICATION

### CLONING PUTATIVE Ste20 SERINE/THREONINE KINASES

PCR in conjunction with the primers SEQ ID NOs. 5-7 was used to amplify sequences from a murine erythroleukemia cDNA library in λgt11. In one reaction, the primer set was SEQ ID NOs. 5 and 6, and in a separate reaction, the primer set was SEQ ID NOs. 5 and 6. In all reactions Taq polymerase was used.

Sequence alignment revealed that C12-2bs and 5e.new share homology with the Sterile 20 family of protein kinases (Figure 4). C12-2bs and 5e.new represent new members of the Sterile 20 family of protein kinases.

## EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

# CLAIMS

What is claimed is:

1. A purified nucleic acid comprising at least 10 continuous nucleotides of a nucleic acid sequence provided in SEQ ID NO.: 1.

2. The purified nucleic acid of Claim 1 comprising 27 continuous nucleotides of said nucleic acid sequence.

3. The purified nucleic acid of Claim 1 comprising at least 45 continuous nucleotides of said nucleic acid sequence.

4. The purified nucleic acid of Claim 1 comprising at least 100 continuous nucleotides of said nucleic acid sequence.

5. A purified nucleic acid comprising at least 10 continuous nucleotides of a nucleic acid sequence provided in SEQ ID NO.: 3.

6. The purified nucleic acid of Claim 5 comprising at least 27 continuous nucleotides of said nucleic acid sequence.

7. The purified nucleic acid of Claim 5 comprising at least 45 continuous nucleotides of said nucleic acid sequence.

8. The purified nucleic acid of Claim 5 comprising at least 100 continuous nucleotides of said nucleic acid sequence.

9. A purified nucleic acid sequence comprising a nucleic acid sequence encoding at least 8 continuous amino acids of a amino acid sequence provided in SEQ ID NO.: 2.

10. The purified nucleic acid of Claim 9 wherein said nucleic acid encodes at least 18 continuous amino acids of said amino acid sequence.

11. The purified nucleic acid of Claim 9 wherein said nucleic acid encodes at least 34 continuous amino acids of said amino acid sequence.

12. The purified nucleic acid of Claim 9 wherein said nucleic acid encodes at least 54 continuous amino acids of said amino acid sequence.

13. The purified nucleic acid of Claim 9 wherein said nucleic acid encodes a serine/threonine kinase.

14. The purified nucleic acid of Claim 9 wherein said nucleic acid encodes a member of the sterile 20 family of proteins.

15. A purified nucleic acid sequence comprising a nucleic acid sequence encoding at least 8 continuous amino acids of a amino acid sequence provided in SEQ ID NO.: 4.

16. The purified nucleic acid of Claim 15 wherein said nucleic acid encodes at least 18 continuous amino acids of said amino acid sequence.

17. The purified nucleic acid of Claim 15 wherein said nucleic acid encodes at least 34 continuous amino acids of said amino acid sequence.

18. The purified nucleic acid of Claim 15 wherein said nucleic acid encodes at least 54 continuous amino acids of said amino acid sequence.

19. The purified nucleic acid of Claim 15 wherein said nucleic acid encodes a serine/threonine kinase.

20. The purified nucleic acid of Claim 15 wherein said nucleic acid encodes a member of the sterile 20 family of proteins.

21. A purified nucleic acid comprising a nucleotide sequence which comprises at least 16 nucleotides hybridizing under stringent conditions to at least 20 continuous nucleotides provided in SEQ ID NO. 1.

22. A purified nucleic acid comprising a nucleotide sequence which comprises at least 18 nucleotides hybridizing under stringent conditions to at least 20 continuous nucleotides provided in SEQ ID NO. 1.

23. A purified nucleic acid comprising a nucleotide sequence which comprises at least 19 nucleotides hybridizing under stringent conditions to at least 20 continuous nucleotides provided in SEQ ID NO. 1.

24. A purified nucleic acid comprising a nucleotide sequence which comprises at least 16 nucleotides hybridizing under stringent conditions to at least 20 continuous nucleotides provided in SEQ ID NO. 3.

25. A purified nucleic acid comprising a nucleotide sequence which comprises at least 18 nucleotides hybridizing under stringent conditions to at least 20 continuous nucleotides provided in SEQ ID NO. 3.

26. A purified nucleic acid comprising a nucleotide sequence which comprises at least 19 nucleotides hybridizing under stringent conditions to at least 20 continuous nucleotides provided in SEQ ID NO. 3.

27. A purified nucleic acid comprising SEQ ID NO. 1.

28. A vector comprising the nucleic acid of Claim 27.

29. A host cell comprising the vector of Claim 28.

30. A method of making the protein encoded by SEQ ID NO. 1 comprising, transfecting a host cell with the vector of Claim 28 and isolating the expressed protein.

31. A protein encoded by the nucleic acid of Claim 27.

32. An antibody that specifically binds to the protein of Claim 31.

33. A purified nucleic acid comprising SEQ ID NO. 3.

34. A vector comprising the nucleic acid of Claim 33.

35. A host cell comprising the vector of Claim 34.

36. A method of producing a serine/threonine kinase comprising, transfecting a host cell with the vector of Claim 34 and isolating the expressed protein.

37. A protein encoded by the nucleic acid of Claim 34.

38. An antibody that specifically binds to the protein of Claim 37.

39. A purified nucleic acid comprising a nucleic acid sequence encoding an amino acid sequence comprising SEQ ID NO. 2.

40. A purified nucleic acid comprising a nucleic acid sequence encoding an amino acid sequence comprising SEQ ID NO. 4.

41. A serine/threonine kinase comprising the amino acid sequence of SEQ ID NO. 2.

42. An antibody that specifically binds to the serine/threonine kinase of Claim 41.

43. A serine/threonine kinase comprising the amino acid sequence of SEQ ID NO. 4.

44. An antibody that specifically binds to the serine/threonine kinase of Claim 43.

C12-2bs. Length: 1504

```
   1  ctagtcgggc ctgccgggca tgcagaacct gaaagcagac ccagaagagc
  51  tttttaccaa gctagagaag attggaaagg gctcttttgg tgaagtgttc
 101  aaaggcattg acaatcggac tcagaaagtg gtggccataa aaatcattga
 151  tctggaagaa gccgaggacg agatagagga catccaacaa gagatcacag
 201  tgctgagcca gtgtgacagt ccctacgtca ccaagtacta tggatcctat
 251  ctcaaggata ctaagttgtg gataatcatg gagtatcttg gtggaggctc
 301  tgccctggat ctgttagagc ctggcccttt agatgaaatt cagattgcaa
 351  ccatattacg agaaattctg aaaggacttg attatctaca ctcggagaag
 401  aaaattcaca gagatattaa agaggccaat gttctgctct ctgaacatgg
 451  agaggtgaag ctggcagact ttggagtggc cggccagctg acggataccc
 501  agatcaaaag gaacaccttc gtgggtaccc ccttctggat ggcgccggag
 551  gtcatcaagc agtcagccta cgactcaaag gcagacatct ggtcccttgg
 601  catcaccgca atagaactgg ccaaaggaga gccaccacat tctgagctgc
 651  accccatgaa ggtgttattc ctcatcccaa agaacaaccc tcccacactg
 701  gaagggaact acagcaaacc cctcaaggag ttcgtggagg cctgcctgaa
 751  caaggagccc agctttaggc ccactgctaa ggaattattg aagcacaaat
 801  tcataatccg caatgcaaag aaaacgtcct acttgaccga gcttatcgac
 851  aggtacaaga ggtggaaggc ggagcagagc cacgaggact ccagctcgga
 901  ggactctGAC GTGGAGACAG ATGGCCAGGC GTCTGGAGGC AGCGACTCTG
 951  GGGACTGGAT CTTCACTATC CGGGAGAAAG ATCCCAAGAA TCTGGAGAAC
1001  GGAACTCTTC AGCTCTCGGA CTTGGAAAGA AATAAGATGA AAGATATCCC
1051  AAAGAAGCCT TTCTCTCAGT GTTTTATCCC ACAATCATTT CTCCTCCTGT
1101  TTTGCGGAGC TGAAAAGACA AAGAGCCAAG GCATGCGGAG GGAACTTGGG
1151  GTCAATAGAA GAGCTGCGGG GGAGCCATCT ACTTGGCGGA AGAGGCCTGC
1201  CCTGGGATCT CAGACACTAT GGTGGCACAG CTTGTGCAGC GGCTGCAGAG
1251  ATATTCTCTG AGTGGCGGAG GAGCCTCAGC GCACTGAAGG CCCATGGCGC
1301  CCGGGTTGGT TTTTCCTTTC TTCTTCATCT TCCTTCTTTT TAAAAGTCAA
1351  CGAGAGCCTT TGCCGACTCT GCGAAGAGGT GTCACGGAGG GGCCCACCCG
1401  CCCTCCCATA GCGCCGGCAC CTGTCCCTCG TGCCGAATTC CTGCAGCCCG
1451  GGGGATCCAC TAGTTCTAGA GCGGCCGCCA CCGCGGTGGA GCTCCAGTTT
```

Fig. 1

5e.New Length: 1979 2/6

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   1  GGCACGAGCC CAGGTCCCAG GCACCGCCAC AGGTCAAGCC CTGCATTCAG
  51  GAAAGAGAGC AACACTGCAG TTAGCCAAAA GCCAGGCAGG CGAGCGGCAT
 101  AGAGGCCTCG ATCGAGAAGC CCGGTAGAGC TGCAGAGATA CCTCCGTAGG
 151  AGGAGCCAGT CTCTGCCGGA GGCGCCACCG CCACCACCGC AGAAGCAGCG
 201  CGAAGTAGCA GTCGCCACCA TGGCCCACTC ACCGGTGGCT GTTCAAGTGC
 251  CTGGGATGCA GAATAATATA GCAGATCCAG AAGAACTGTT CACAAAATTA
 301  GAGCGCATTG GAAAAGGCTC CTTTGGAGAA GTTTTCAAAG GAATTGATAA
 351  CCGTACTCAG CAAGTGGTTG CAATTAAAAT CATTGACCTT GAGGAAGCTG
 401  AGGATGAAAT AGAAGACATC CAACAAGAAA TAACTGTTTT GAGTCAGTGC
 451  GACAGCTCAT ATGTAACAAA ATACTATGGG TCCTATTTAA AGGGTTCAAA
 501  ACTATGGATA ATAATGGAAT ACCTAGGTGG AGGTTCAGCA TTGGATCTTC
 551  TGCGTGCTGG TCCATTTGAT Gagttccaga ttgccaccat gctcaaggag
 601  attttgaaag gtctggacta tctacattct gaaaagaaaa tccaccgaga
 651  cattaaagct gCcaacgtct tgctttcaGA ACAAGGTGAT GTTAAACTGG
 701  CTGACTTTGG AGTTGCTGGC CAGCTGACAG ATACACAAAT CAAAAGAAAC
 751  ACCTTCGTAG GGACTCCGTT Ttggatggct cctgaagtta ttcaacagtc
 801  agcttatgac tctaaagctg acatatggtc tttgggaatt actgctattg
 851  aacttgccaa gggagagcct Ccgaattctg acatgcatcc aatgagagtt
 901  ctGTTTCTTA TTCCAAAAAA CAACCCTCCA ACTCTTATTG GAGACTTTAC
 951  TAAGTCTTTC AAGGAGTTTA TTGATGCTTG CCTGAATAAA GACCCGTCAT
1001  TTCGTCCTAC AGCTAAAGAA CTTTTGAAGC ATAAGTTCAT CGTAAAAAAT
1051  TCAAAGAAGA CTTCTTATCT GACTGAATTG ATCGATCGAT TTAAGAGATG
1101  GAAGGCAGAA GGCCACAGTG ATGAGGAATC TGATTCCGAG GGCTCTGACT
1151  CGGAATCCAG CAGCAGGGAA AGTAACCCTC ACCCTGAATG GAGTTTCACC
1201  ACTGTGCGTA AGAAGCCTGA TCCAAAGAAA CTGCAGAATG GGGAAGAGCA
1251  AGATCTTGTG CAAACCTTGA GCTGTTTGTC TATGATAATC ACACCTGCAT
1301  TTGCCGAACT TAAACAGCAG GACGAGAATA ATGCGAGTCG AAACCAGGCA
1351  ATTGAAGAAC TTGAGAAAAG TATTGCTGTG GCTGAAACCG CCTGTCCTGG
1401  CATCACAGAT AAGATGGTGA AGAAACTAAT CGAAAAATTT CAAAAGTGTT
1451  CTGCGGATGA ATCCCCTTAA GAAATCTGTT GTCATTACTT TTGGCTTCTG
```

Fig. 2A

1501 TTTCATGTGG ACCAGGAGAA ACCCACCAAA GCTATGTCAA CCTTATAAAT

1551 GCTTAACTCA TGAGCTCCAT GTgccttttg gatctttgcc acattgaaga

1601 tttagaggaa gctattaaac tattttgtga tggtgattat cattttgtat

1651 tttaaagaga ttattttgta aggaataatt ttaatactat agttttgccg

1701 gtattgtagt aaatgctgag atacaggttt tttgtttttt gttttttaat

1751 tttaggtacC AtTAtTTCtT ATGtTCATgG aATGaATACT GtTTgGtTTg

1801 GaATCtTTAG TTAACTGTAT ACTCATaAAC ATACAGGTCt TTCAAAGTCA

1851 TCCTAACTAT TAAATGTtTG TAAATCATCA AGCTtCAAAA agCAtTCttt

1901 ttCCCCcaca caagtatatt ctaaaaagac tatttgtaat gaggtggaag

1951 taagtaatac cttcttaaaa cctcgtgcc

Fig. 2B

SEQ ID No. 3:

5' ATA GGA TCC CA(CT) (AC)G(AGCT) GA(AT) AT(ACT) AA(AG) GG(AGCT) GC(AGCT) AA(CT) AT(ACT) (CT)T 3'

SEQ ID No. 4:

5' TCG GAA TTC (CT)TC (AGCT)GG (AGCT)GC CAT CCA (AG)TA 3'

SEQ ID No. 5:

5' TCG GAA TTC (CT)TC (AGCT)GG (AGCT)GC CAT CCA (AG)AA 3'

Fig. 3

```
      1                                                        50
12-2  .......... .........MQ NLKADPEELF TKLEKIGKGS FGEVFKGIDN
 5e   ......MAHS PVAVQVPGMQ NNIADPEELF TKLERIGKGS FGEVFKGIDN
S201  METVQLRNPP RRQLKKLDED SLTKQPEEVF DVLEKLGEGS YGSVYKAIHK

      51                                                       100
12-2  RTQKVVAIKI IDLEEAEDEI EDIQQEITVL SQCDSPYVTK YYGSYLKDTK
 5e   RTQQVVAIKI IDLEEAEDEI EDIQQEITVL SQCDSSYVTK YYGSYLKGSK
S201  ETGQIVAIKQ VPV...ESDL QEIIKEISIM QQCDSPHVVK YYGSYFKNTD

      101                                                      150
12-2  LWIIMEYLGG GSALDL..LE PGPLDETQIA TILREILKGL DYLHSEKKIH
 5e   LWIIMEYLGG GSALDL..LR AGPFDEFQIA TMLKEILKGL DYLHSEKKIH
S201  LWIVMEYCGA GSVSDIIRLR NKTLTEDEIA TILQSTLKGL EYLHFMRQIH

      151                                                      200
12-2  RDIKEANVLL SEHGEVKLAD FGVAGQLTDT QIKRNTFVGT PFWMAPEVIK
 5e   RDIKAANVLL SEQGDVKLAD FGVAGQLTDT QIKRNTFVGT PFWMAPEVIQ
S201  RDIKAGNILL NTEGHAKLAD FGVAGQLTDT MAKRNTVIGT PFWMAPEVIQ

      201                                                      250
12-2  QSAYDSKADI WSLGITAIEL AKGEPPHSEL HPMKVLFLIP KNNPPTL..E
 5e   QSAYDSKADI WSLGITAIEL AKGEPPNSDM HPMRVLFLIP KNNPPTL..I
S201  EIGYHCVADI WSLGITAIEM AEGKPPYADI HPMRAIFMIP TNPPPTFRKP

      251                                                      300
12-2  GNYSKPLKEF VEACLNKEPS FRPTAKELLK HKFIIRNAKK TSYLTELID.
 5e   GDFTKSFKEF IDACLNKDPS FRPTAKELLK HKFIVKNSKK TSYLTELID.
S201  ELWSDNFMDF VKQCLVKSPE QRATATQLLQ HPF.VKSAKG VSILRDLINE

      301                                                      350
12-2  ....RVKR.. ...WKAE.QS HEDSSSEDSD VETDGQASGG SDSGDWIFTI
 5e   ....RFKR.. ...WKAEGHS DEESDSEGSD SESSSRESNP HPEWSFTTVR
S201  AMDVKLKRQE AQQPEVDQDD EENSEEDEMD SGTMVRTAGD EMGTVRVAST

      351                                                      400
12-2  REKDPKQL.. .......... ......ENGT LQLSDLEPNK MKDIPKHPFS
 5e   KKPDPKKL.. .......... ......QNGE EQ........ ........DLV
S201  MSGGANTMIE HGDTLPSQLG TMVINTEDEE EEGTMKRRDE TMQPAKPSFL

      401                                                      450
12-2  QCFIPQSFLL LFCGAEKTKS QGMPRELGVN RRAAGEPSTW RKRPALGSQT
 5e   QTLSCLSMII TPAFAE.... ..LKQQDENN ASRNQAIEEL EKSIAVA...
S201  EYFEQKEKEN QINSFGKNVS GSLKNSSDWK IPQDGDYEFL KSWTVEDLQK

      451                                                  496
12-2  LWWHSLCSGC RDIL..... .......... .......... ......
 5e   ...ETACPGI TDKMVKKLIE KFQKCSADES P......... ......
S201  RLL.....AL DPMMEQEMEE IRQKYRSKRQ PILDAIEAKK RRQQNF
```

Fig. 4

SUBSTITUTE SHEET (RULE 26)

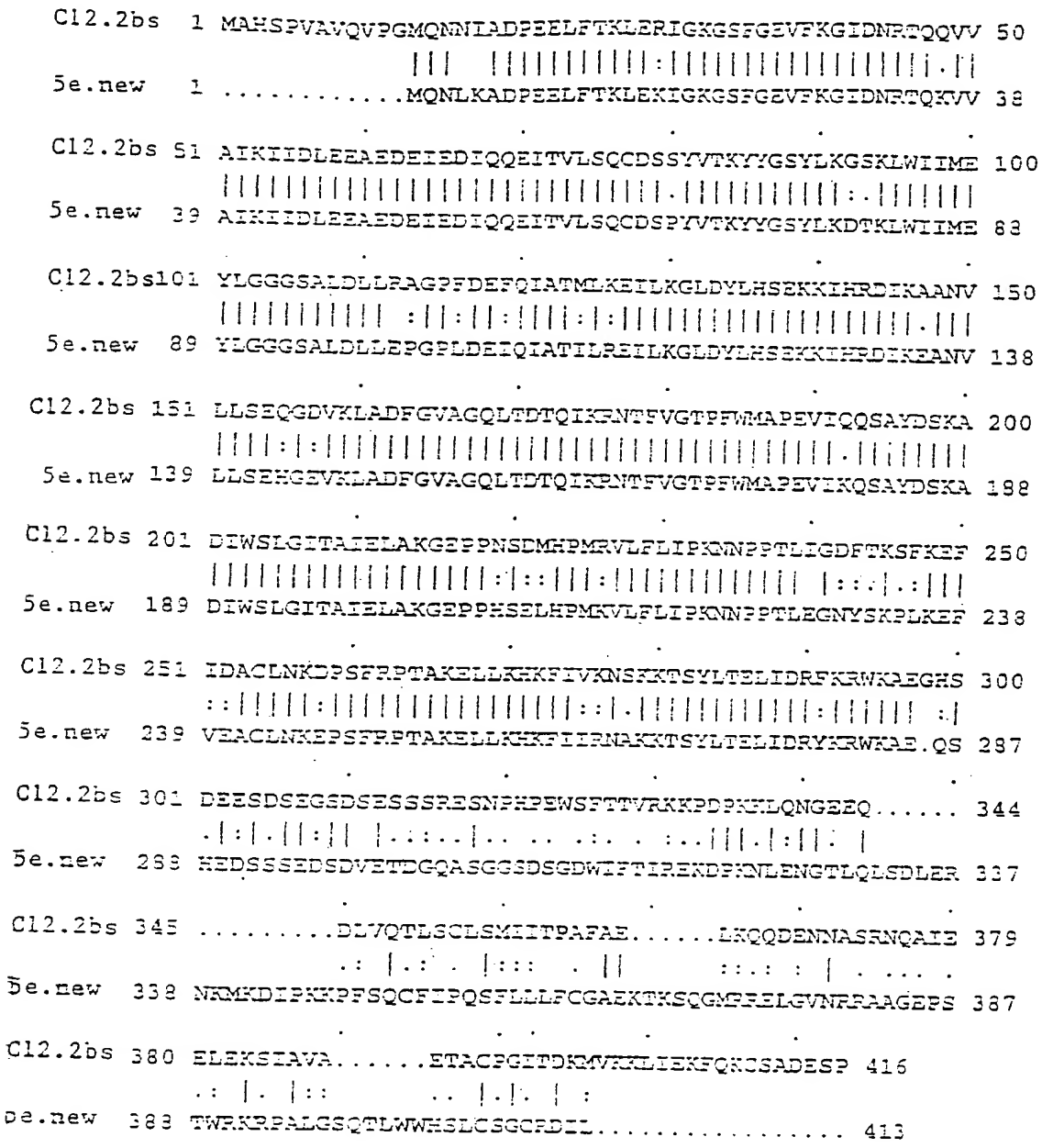


Fig. 5.

# INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 98/26116

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N9/12 C07K16/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | DATABASE EMBL - EMEST1<br>Entry/Acc.no. Aa563280, 11 September 1997<br>MARRA M. ET AL.: "v155a08.r1 Stratagene<br>mouse skin (#937313) Mus musculus cDNA<br>clone 976118 5' similar to WP:T19A5.2<br>CE07510 SERINE.THREONINE KINASE."<br>XP002098719 | 1-4,<br>9-14,<br>21-23 |
| Y | see the whole document | 27-31,<br>39,41 |

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[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 April 1999 | 16/04/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Smalt, R |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Inte ›nal Application No
PCT/US 98/26116

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | SCHINKMANN ET AL: "Cloning and characterization of a human STE20-like protein kinase with unusual cofactor requirements" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 45, 7 November 1997, pages 28695-28703, XP002086764 | 1,2,5, 9-17, 19-26, 32,38, 42,44 |
| Y | see page 28696, left-hand column, paragraph 6; figures 1,2,4 | 27-31, 39,41 |
| X | WO 97 42212 A (GEN HOSPITAL CORP) 13 November 1997 see page 15, line 20 see page 17, paragraph 3; figures 1,2 | 1,5, 9-11, 13-26, 32,38, 42,44 |
| X | OSADA S, IZAWA M, SAITO R, MIZUNO K, SUZUKI A, HIRAI S, OHNO S: "YSK1, a novel mammalian protein kinase structurally related to Ste20 and SPS1, but is not involved in the known MAPK pathways." ONCOGENE, vol. 14, no. 17, 1 May 1997, pages 2047-57, XP002098718 see page 2055, left-hand column, paragraph 5; figures 1-3 | 1,5, 9-11, 13-17, 19-26, 32,38, 42,44 |
| X | DATABASE EMBL - EMEST13 Entry Hs504336, Acc.no. W16504, 4 May 1996 HILLIER L. ET AL.: "zb05e11.r1 Soares fetal lung NbHL19W Homo sapiens cDNA clone 301196 5' similar to SW:KPAK_RAT P35465 SERINE/THREONINE-PROTEIN KINASE PAK" XP002098720 see the whole document | 5,6,15, 19,24-26 |
| P,X | DATABASE EMBL - EMEST4 Entry/Acc.no Aa881667, 30 March 1998 MARRA M. ET AL.: "vx21d04.r1 Soares 2NbMT Mus musculus cDNA clone 1265095 5' similar to TR:O14840 O14840 STE20-LIKE KINASE 3. " XP002098721 see the whole document | 5-8, 15-20, 24-26 |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 26116

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/ US 98 /26116

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-4,9-14,21-23,27-32,39,41,42
   Nucleic acid encoding a first Ste20 homolog designated c12.2bs, fragments thereof, vector comprising said nucleic acid, host transformed with said nucleic acid, method of producing c12.2bs protein, the protein itself, and antibodies against said protein.

2. Claims: 5-8,15-20,24-26,33-38,40,43,44
   Nucleic acid encoding a second Ste20 homolog designated 5e.new, fragments thereof, vector comprising said nucleic acid, host transformed with said nucleic acid, method of producing 5e.new protein, the protein itself, and antibodies against said protein.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 98/26116

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9742212 A | 13-11-1997 | AU 3118297 A | 26-11-1997 |